AD_____

SULFUR MUSTARD (SM) LESIONS IN ORGAN-CULTURED HUMAN SKIN: MARKERS OF INJURY AND INFLAMMATORY MEDIATORS (U)

ANNUAL REPORT

ARTHUR M. DANNENBERG, JR.

MARCH 1, 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7040

Department of Environmental Health Sciences
The Johns Hopkins University
School of Hygiene and Public Health
615 N. Wolfe Street
Baltimore, Maryland 21205-2179

Approved for public release, distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: unclassified	1b. RESTRICTIVE MARKINGS: n.a.
2a. SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION / AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE: n.a.	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: The Johns Hopkins University School of Hygiene & Pub. Hlth; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): 615 North Wolfe Street, Baltimore, Maryland 21205-2179	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-87-C-7040
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62734A	3M16 2734A875	AA	377

11. TITLE (Include Security Classification): SULFUR MUSTARD (SM) LESIONS IN ORGAN-CULTURED HUMAN SKIN: MARKERS OF INJURY AND INFLAMMATORY MEDIATORS (U)

12. PERSONAL AUTHOR(S): Dannenberg, Arthur M., Jr.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 02/17/87 TO 02/16/88	March 1, 1988	45

16. SUPPLEMENTARY NOTATION

17 COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Sulfur mustard; human skin lesions; histology & histochemistry; inflammatory mediators; organ culture; viability of human skin at 4 C; paranuclear vacuolization test for (cont'
06	01		
06	11		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

In this research program, we are (a) developing an in vitro human skin model with which to assess the amount of injury produced by topically applied sulfur mustard (SM), and (b) identifying the early mediators of the inflammatory response produced by SM in human skin.

(a) The most promising marker of skin injury is our paranuclear vacuolization test. SM was applied in various dilutions to full-thickness 1.0-cm^2 human skin explants. The explants were incubated at 37 C for 24 hr, histologic sections were made, and the number of vacuoles determined microscopically. Practical details, such as dose-response curves, and the length of time human skin can be stored at 4 C and still be of use in this test, have also been determined. This test can now be used on human skin explants to assess the efficacy of protective ointments and decontaminating agents.

(continued on next page)

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mrs. Virginia Miller	301-663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

18. SUBJECT TERMS (continued from page 1 of DD FORM 1473)

epidermal injury; [^{14}C]leucine incorporation; chemotaxis of granulocytes and macrophages; lysosomal enzymes of skin; acid phosphatase, B-glucuronidase, B-galactosidase, lysozyme (lactic dehydrogenase); trypsin-like and chymotrypsin-like enzymes; angiotensin-converting enzyme; plasminogen activator.

19. ABSTRACT (continued from page 1 of DD FORM 1473)

A second, but more cumbersome, way to assess injury to human skin explants is the interference with the incorporation of [^{14}C]leucine by the epidermal cells of the explant. Again, dose-response relationships were determined.

(b) Culture fluids of human skin explants to which SM was topically applied were assayed for markers of cell death and early inflammatory mediators. Lactic dehydrogenase (LDH), angiotensin-converting enzyme (ACE), trypsin-like and chymotrypsin-like proteases, and lysosomal enzymes (acid phosphatase β-glucuronidase, β-galactosidase, and lysozyme) were present. The culture fluids from both SM-treated explants and control explants showed similar concentrations of these enzymes. Therefore, these enzymes could not be used as markers for injury produced by SM. ACE is a marker for endothelial damage. Lysosomal enzymes participate in cell autolysis.

We did, however, find one type of early inflammatory mediator that was increased by SM: Greater amounts of plasminogen-activating proteases were released in culture by SM-treated human skin explants than by control explants. Such plasminogen activators are known to be associated with blister formation.

Identification of other phlogistic factors, associated with human skin injury by SM, will be made during the next year of our contract.

AD_____

SULFUR MUSTARD (SM) LESIONS IN ORGAN-CULTURED HUMAN SKIN: MARKERS OF INJURY AND INFLAMMATORY MEDIATORS (U)

ANNUAL REPORT

ARTHUR M. DANNENBERG, JR.

MARCH 1, 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7040

Department of Environmental Health Sciences
The Johns Hopkins University
School of Hygiene and Public Health
615 N. Wolfe Street
Baltimore, Maryland 21205-2179

SUMMARY

In this research program, we are (a) developing an *in vitro* human skin model with which to assess the amount of injury produced by topically applied sulfur mustard (SM), and (b) identifying markers of cell death and early mediators of the inflammatory response produced by SM in human skin.

(a) The most promising marker of skin injury is our paranuclear vacuolization test. SM was applied in various dilutions to full-thickness 1.0-cm^2 human skin explants The explants were incubated at 37 C for 24 hr, histologic sections were made, and the number of vacuoles determined microscopically. Practical details, such as dose-response curves, and the length of time human skin can be stored at 4 C and still be of use in this test, have also been determined. This test can now be used on human skin explants to assess the efficacy of protective ointments and decontaminating agents.

A second, but more cumbersome, way to assess injury to human skin explants is the interference with the incorporation of [^{14}C]leucine by the epidermal cells of the explant. Again, dose-response relationships were determined.

(b) Culture fluids of human skin explants to which SM was topically applied were assayed for markers of cell death and early inflammatory mediators. Lactic dehydrogenase (LDH), angiotensin-converting enzyme (ACE), trypsin-like and chymotrypsin-like proteases, and lysosomal enzymes (acid phosphatase, β-glucuronidase, β-galactosidase, and lysozyme) were present. The culture fluids from both SM-treated explants and control explants showed similar concentrations of these enzymes. Therefore, these enzymes could not be used as markers for injury produced by SM. ACE is a marker for endothelial damage. Lysosomal enzymes participate in cell autolysis.

We did, however, find one type of early inflammatory mediator that was increased by SM: Greater amounts of plasminogen-activating proteases were released in culture by SM-treated human skin explants than by control explants. Such plasminogen activators are known to be associated with blister formation.

Identification of other phlogistic factors, associated with human skin injury by SM, will be made during the next year of our contract.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978)

TABLE OF CONTENTS

continued

FIGURES:

TABLES:

INTRODUCTION

Full-thickness explants of discarded human skin survive well in organ culture (1). The skin can be obtained from recent autopsies and from a variety of surgical procedures. The cultured skin explants can be used to assess the toxicity of topically applied chemicals. For this purpose, our paranuclear vacuolization test (1) has proven to be most practical, although our more time-consuming [^{14}C]leucine incorporation test (1) is quite satisfactory.

This report describes the application and development of these methods, so that they could be used to screen prophylactically applied ointments used to protect human skin against sulfur mustard (SM) injury. We determined (a) the length of time human skin can be kept at 4 C and still remain viable and useful for testing chemical toxicity, (b) the dose-response curve in our paranuclear vacuolization test for different concentrations of SM applied to human skin, and (c) whether a variety of enzymatic and biological assays could be useful in assessing chemical toxicity to human skin.

We also assayed some of the markers of cell death and some of the early mediators released into culture fluids from full-thickness human skin explants exposed *in vitro* to SM.

MATERIALS AND METHODS

Organ culture of 1.0-cm^2 skin specimens (1)

The skin specimens were clipped free of hair (if hair was present) and then wiped lightly with 70% alcohol. They were placed on a sterile plastic sheet, made wet with Hanks' balanced salt solution (GIBCO Laboratories, Grand Island, NY, Cat. No., 310-4025) containing penicillin (1000 U/ml) and streptomycin (1000 ug/ml), and the subcutaneous fat was removed with scissors. Gloves and a surgical mask were worn, and these procedures were performed in a hood.

The skin was cut precisely into 1.0-cm^2 full-thickness pieces and washed three times with the antibiotic-containing Hanks' solution. Then, each explant was placed in a small, sterile, plastic Petri dish (35 x 10 mm, Falcon Plastics, Division of Becton Dickinson Co., Oxnard, CA). The epidermis was patted dry with sterile surgical gauze, and two drops of RPMI 1640 culture medium was added to the Petri dish in order to keep the dermal (underneath) side of the explant moist. Then, under a forced-draft hood, we spread, by means of a Hamilton syringe, 10 ul of dilute SM (or its vehicle) over the entire (dry) upper surface of the explant. Our standard concentration of SM is 1.0% in methylene chloride, but 0.01 to 1.0% was used for the various studies herein reported.

SM was applied in a stainless steel hood with a face draft of 150 linear feet of air per minute. The specimens were left 30 to 40 min at room temperature in the hood before they were organ-cultured.

Our culture medium (2.0 ml/Petri dish) was composed of medium RPMI 1640 containing glutamine (GIBCO Laboratories, Cat. No. 320-1875), supple-

mented with penicillin (100 U/ml), streptomycin (100 ug/ml), and additional glutamine (2.0 mM). The final concentrations are in parentheses. For the [^{14}C]leucine incorporation experiments, [^{14}C]leucine (0.25 uCi/ml, 350 mCi/mmol) was included.

Three small Petri dishes, each containing an explant, were then placed in one large Petri dish (100 x 15 mm, Falcon Plastics), and the large Petri dishes were stacked in a heavy plastic vacuum jar (Oxoid U.S.A., Columbia, MD). The jar was gassed with a 95% O_2--5% CO_2 mixture at 1.1 to 1.2 atmospheres of pressure, and the jar was sealed. It was rocked six times per minute in an incubator at 37 C for 24 hr. The tops of the 1.0-cm^2 skin explants were not covered by the culture medium, but were exposed directly to the gaseous O_2/CO_2 mixture. They did, however, become moist (see 1).

Preparation of 1- to 2-um glycol methacrylate-embedded tissue sections (1,2)

Water-soluble methacrylate. The JB-4 kit (Polysciences, Inc., Warrington, PA) was used. The kit contains three components: Solution A (glycol methacrylate, modified with 2-butoxyethanol), Solution B (the accelerator, N,N-dimethylaniline in polyethylene glycol 400), and Catalyst C (benzoyl peroxide). Directions for their use are provided with each kit.

Fixation. A 10% buffered formalin was obtained from Columbia Diagnostics, Springfield, VA. This product contains 3.7 to 4.0% technical grade formaldehyde, 18.6 g/L monobasic sodium phosphate, 4.2 g/L sodium hydroxide, and about 1% methanol. It has a pH of 7.2 and a buffer osmolality of 290 mOsm/kg. A modified Karnovsky's formulation (2) was prepared by mixing 100 ml of 50% glutaraldehyde (Polysciences, Inc.), 25 ml of glycerol, and 900 ml of the 10% buffered formalin. The 1.0-cm^2 skin explants were placed in this fixative at 4 C for 1 or 2 days.

Embedding (3). The fixed tissue samples were washed at 4 C for 24 hr in 0.05 M potassium phosphate buffer, pH 7.2. (The samples may be left in this cold buffer for several days.) They were dehydrated for 2 hr each in 50%, 70%, 95%, and 95% ethanol (all containing 2.5% glycerol), after which they were infiltrated 1 to 3 days at 4 C in Solution A with catalyst added.

A molding tray containing multiple 6 x 5 x 12 mm wells (Polysciences, Inc., Warrington, PA) was placed in a shallow basin of cracked ice. The bottom of each well was covered with catalyzed Solution A containing 5% Solution B (i.e., a 1:20 ratio of B:A), prepared with cold (4 C) reagents. Then each Solution A-infiltrated explant was cut through its center (perpendicular to its epidermal surface) and its cut surface carefully placed flat against the bottom of a well in the molding tray. The wells were filled with the catalyzed Solution A and B mixture (described above), and aluminum JB-4 block holders (Polaron Instruments, Inc., Division of BioRad, Cambridge, MA) were placed on the top of each well. Slight overfilling of the wells eliminated the need for special measures to exclude oxygen. The entire basin was placed in a 4 C coldroom, and the glycol methacrylate in the molding tray was allowed to polymerize overnight. Then the blocks were removed from the tray and examined for hardness. If too soft, they were placed in a desiccator at room temperature until adequately hardened.

Coating microscope slides. Microscope slides were sprayed lightly (in a hood) with a fine mist of Solution A. Care was taken to avoid coalescence of the droplets on the slides. They were promptly placed in an oven at about 80 C and dried (usually overnight). (Note: The noxious vapors released during drying should not be inhaled.) Then, this spraying-drying sequence was repeated a second time.

Sectioning. The JB-4 microtome (DuPont Instruments, Norwalk, CN) was used with Ralph-type glass knives. The knives were made on a Histo-Knife-maker (LKB Instruments, Rockville, MD) from 38-mm glass strips, broken 3 to 4 mm from the score at an intermediate fulcrum setting.

The blocks were faced until the full width of tissue was just encountered. Sections were then cut at 2 um. Each section was straightened as much as possible while on the knife, transferred to an elliptical pool of water on a precoated slide, and allowed to spread. Then the water was carefully blotted away, and the slide was dried at room temperature. (Sections spread better when the water contains about 1% ammonium hydroxide.)

Staining (3). Ten milliliters of 0.2 M Tris-maleate buffer was diluted with 40 ml of water and adjusted to pH 5.4 with 1 N NaOH (see 3). Five milliliters of Giemsa concentrate (Harleco, Cat. No. 620, Gibbstown, NJ) was added, and the mixture was stirred vigorously for at least 10 min. The stain was then filtered two times into a standard Coplin jar, each time through fresh filter paper (Grade 588 for coarse precipitates, Schleicher and Schuell, Inc., Keene, NH). Fresh working stain was prepared daily. The Giemsa concentrate (kept at room temperature) could be used satisfactorily for at least 6 months.

The plastic sections were stained in the Coplin jar for 60 min, rinsed briefly in a jar of distilled water, dried immediately in a warm air stream, and mounted under a coverslip in a methacrylate medium (Flo-Texx, Lerner Laboratories, Stamford, CN).

Glycol methacrylate-embedded tissue sections are easier to prepare than paraffin-embedded sections. Glycol methacrylate (GMA) is more hydrophilic than paraffin. Therefore, tissue specimens do not need to be completely dehydrated for embedding, nor do the resulting sections need to be rehydrated before staining.

Counting paranuclear vacuoles. All paranuclear vacuoles seen microscopically with a 40 X objective lens and a 10 X eye piece were counted in the entire epidermis of the 1.0-cm explant. A photograph of ten such vacuoles appears in reference 1.

Viability of the explants.

Histologic evaluation is one of the best ways to determine cell and tissue viability in organ-cultured explants (personal communication of Dame Honor B. Fell, Strangeways Laboratories, Cambridge, England). The human skin explants survived well in organ culture for at least 7 days, and there was no morphologic evidence of epidermal cell death in normal skin explants.

Measurement of [^{14}C]leucine incorporation into proteins of the skin explants (1)

Full-thickness skin explants were organ-cultured for 24 hr at 37 C in medium RPMI 1640 containing [^{14}C]leucine, as described above in the first section of MATERIALS AND METHODS, called "Organ culture of 1.0-cm^2 skin specimens (1)."

After incubation, the radioactive supernatant fluid was decanted, and each section was washed twice with 2 ml cold RPMI 1640. They were then transferred to a Petri dish, minced with two razor blades, placed in a glass homogenizer (with a motor-driven Teflon pestle) in a bucket of ice, and homogenized for 10 min in 3.0 ml of 20% trichloroacetic acid (TCA). The tissue suspension was transferred to a 18 x 100 mm Sorvall centrifuge tube with 3.0 ml of 5% TCA and centrifuged for 15 min at 10,000 rpm. The supernatant fluid was discarded, and the pellet was resuspended in another 3.0 ml of 5% TCA. This washing procedure was repeated a total of four times, so that almost all of the TCA-extractable radioactivity was removed. Upon completion of the last centrifugation, the sediment was transferred to a glass scintillation vial with 5 ml of anhydrous ether/aceton/chloroform (2:2:1) mixture, shaken, and incubated at 50 C for 30 min to extract the lipids. The solvent was discarded, 1.0 ml of 0.5 M NaOH was added, and the specimens were incubated at 80 C for 30 min. Then 2.0 ml of Protosol (New England Nuclear Research Products, Boston, MA, Cat. No. NEF-935) was added and the solubilization of the tissue specimens continued overnight on a rocker platform at 37 C. Eight milliliters of liquid scintillation counting fluid (Aquasol-2, New England Nuclear Research Products, Cat. No. NEF-952) was added to each vial. Then the amount of [^{14}C]radioactivity was read in a scintillation counter.

Lysosomal enzymes, lactic dehydrogenase (LDH) and protein released in vitro by full-thickness human skin explants

Culture fluids from 1.0-cm^2 full-thickness human skin explants (exposed in vitro to SM) were assayed for acid phosphatase, β-glucuronidase, β-galactosidase, lysozyme, LDH, and total protein (4). The assay methods are also described in Chapter 5 of reference 5.

Proteases released in vitro by full-thickness human skin explants

Culture fluids from 1.0-cm^2 full-thickness human skin explants, exposed in vitro to SM, were assayed for trypsin-like and chymotrypsin-like proteases with the synthetic peptide substrates, L-leucyl-glycyl-L-arginyl-aminofluorocoumarin (LGA-AFC) (6-9) and N-benzoyl-D,L-phenylalanine-β-naphthyl ester (BPN) (9-11). The assay methods are also described in Chapter 6 of reference 5.

Plasminogen activator

A most sensitive and specific plasminogen activator assay is that of Edward Reich's group (12), used by the Gerald S. Lazarus group at the University of Pennsylvania (13,14). (Dr. Pamela J. Jensen of the latter group guided us in establishing the method in our own laboratory.)

Fibrinogen (Sigma Chemical Co., St. Louis, MO, Cat. No. F-4883), 2.0 mg in 2.0 ml 0.1 M sodium phosphate buffer (pH 7.0), was placed in a plastic

15-ml centrifuge tube in an ice bath. Sodium ^{125}iodide (1000 uCi in 10 ul) from Amersham International Plc, Amersham, UK (Cat. No. IMS.30, carrier-free, 100 mCi/ml) was neutralized with 10 ul 0.1 N HCl and was added to the fibrinogen preparation. Then 400 ul chloramine-T (Sigma, Cat No. C-9887) (5 mg in 10 ml of the phosphate buffer) was added. The mixture was vortexed for 2 min. Then 400 ul sodium metabisulfite ($Na_2S_2O_5$) (5 mg in 10 ml of the phosphate buffer) was added and the vortexing continued for 1 min.

In 1.5-ml conical microcentrifuge tubes with a hole in the bottom (made by a 25-gauge needle) plugged by siliconized glass wool, 16 minicolumns of Sephadex G-25 were prepared in the 0.1 M phosphate buffer. The columns were centrifuged (to remove the buffer) at 1500 rpm (200 x g) for 1 min in a 12 x 75 mm polypropylene tube with a hole near its top. Then, to prevent nonspecific blockage of ^{125}I-fibrinogen, unlabeled fibrinogen (0.2 ml, 1.0 mg/ml) was applied to each column. The minicolumns were centrifuged again and the eluate discarded.

The ^{125}I-labeled fibrinogen mixture (about 200 ul) was applied to each of the 16 columns and the centrifugation repeated. The eluates were collected, pooled, and diluted to 8.0 ml with the phosphate buffer.

The ^{125}I-fibrinogen was adjusted to 4 x 10^5 cpm/ml with unlabeled fibrinogen (0.1 mg/ml of buffer, diluted with distilled water 1:11). To each 1.8-cm diameter well of a 96-well plastic sheet, 250 ul of the ^{125}I-fibrinogen mixture was added (about 0.1 uCi/well). The wells were dried at 37 C for about 2 days.

Measurement of total releasable radioactivity. Thrombin (Sigma Chemical Co., Cat. No. T-6884, from human plasma), 300 ul (1 unit/ml), was added to each well, and the thrombin--^{125}I-fibrinogen mixture was incubated 1.5 hr at 37 C. The supernatant fluids were harvested and 200 ul counted in a scintillation counter. The wells were washed twice gently with the buffer. Then 300 ul of trypsin from bovine pancreas (Sigma, Cat. No. T-8253) (1.0 mg/ml in 0.1 M Tris-HCl (pH 7.6)) was added to each well. After the reactants were incubated at 37 C for 1.5 hr in a humidified incubator, the supernatant fluids (200 ul) were harvested and counted in the scintillation counter. There were 20,000 to 40,000 cpm in the 200 ul. About 15 to 25% of this radioactivity was releasable by thrombin, and 75 to 85% was releasable by trypsin.

Standard curves made with urokinase. After treating the dried fibrinogen with thrombin and washing (as just described), 200 ul Tris-HCl buffer (0.1 M, pH 8.1), 50 ul plasminogen (25 ug/ml) (Sigma, Cat. No. P-1517), and 50 ul human urokinase (Calbiochem-Behring Corp., La Jolla, CA, Cat. No. 672123) were added to each well. The urokinase was diluted in 0.1 M Tris-HCl (pH 8.1), containing 0.1% Triton X-100 (Sigma, Cat. No. T-6878) and 0.25% gelatin (Sigma, Cat. No. G-2500, from swine skin) to make a series containing 0 to 10.0 milli-Ploug units (15) per ml. After the reactants were incubated at 37 C for 1.5 hr, the supernatant fluids were harvested (in 200 ul amounts) and counted in a gamma-counter. Over the entire range tested, a straightline relationship existed between the Ploug units of urokinase and the ^{125}I released.

Assay procedure for plasminogen activator in culture fluids of skin explants. The supernates from centrifuged organ-culture fluids (from human skin explants exposed in vitro to 1.0% SM or to the methylene chloride

diluent) were assayed as just described for urokinase: 50-ul aliquots of culture fluid were used, instead of 50 ul of urokinase.

Reagents for the plasminogen activator assay. Thrombin: 250 units (lyophilyzed) was mixed with 5 ml of distilled water (=50 units/ml) and then diluted 1:50 in Dulbecco's modified Eagle's medium. (Our RPMI 1640 medium should also be satisfactory.) Urokinase in distilled water: 1700 units (lyophilyzed) in the vial was mixed with 1.7 ml of distilled water (=1000 units/ml). A 1:100 dilution was equal to 10 milli-Ploug units/ml, which was diluted to 1.0 to 10 milli-Ploug units/ml to make our standard curve.

Angiotensin-converting enzyme (ACE)

This enzyme is released when vascular endothelial cells are damaged. SM is known to damage dermal microvasculature because it produces a markedly edematous response (16).

The fluorimetric assay of Carmel et al. (17) and Carmel and Yaron (18) was used. Ortho-amino-benzoylglycyl-p-nitro-L-phenylalanyl-L-proline (ABz-Gly-Phe(NO_2)-Pro) was obtained from Sigma Chemical Co. (Cat No. A-4408). After dissolving in 0.4 ml of methanol, it was made into a 1.16 mM stock solution (14 mg in 25 ml 0.2 M Tris-HCl buffer (pH 8.2) containing 1.0 M NaCl). This stock solution was diluted 1:1 in the same Tris-NaCl buffer for the ACE assay, or 1:1 with 0.1 M disodium ethylenediaminetetraacetate (EDTA) solution (in the Tris-NaCl buffer), which serves as a zero time control. (ACE is a zinc-requiring enzyme. The removal of zinc, by EDTA, from the skin culture fluids stops ACE activity.)

Assay procedure. Full-thickness human skin explants (1.0 cm^2) were exposed topically in vitro to 1% SM or its diluent $MeCl_2$ and incubated for 3 to 20 hr in RPMI 1640 in an atmosphere of 95% O_2--5% CO_2, as described above. The culture fluids were centrifuged and stored at -70 C until assayed for ACE.

For this assay, these fluids were thawed and added in 100-ul amounts to either 200 ul of the preincubated substrate solution or 200 ul of the preincubated zero-time control solution. After 45 min of incubation at 40 C, the reaction was stopped by the addition of 3.0 ml of 0.1 M EDTA in the Tris-NaCl buffer. The fluorescence was read in a 4.2-ml cuvette (1.0 cm across) at room temperature in a Perkin-Elmer fluorescence spectrophotometer at a 360 nm excitation wavelength and a 410 nm emission wavelength. The fluorescent intensity was normalized using a standard solution of 2.5×10^{-7} M quinine sulfate in 0.1 M H_2SO_4.

The difference between the fluorescence in the substrate solution and in the substrate-EDTA solution (zero-time control) is a measure of the units of enzyme activity. The units can be converted to micromoles of amino-benzoylglycine (ABz-Gly) released from the substrate by means of a quinine sulfate standard (see 17). One unit is equivalent to 1.5 nmol of ABz-Gly in the 3.3-ml sample read in the fluorometer. Quinine has a fluorescence 5.4 times that of ABz-Gly (17). (Under our experimental conditions, the quinine standard (3.3 ml) shows a fluorescence 3.0 units greater than its solvent (0.1 M H_2SO_4).)

EXPERIMENTAL DATA AND COMMENTS

Dose-response curve: Number of paranuclear vacuoles vs. concentration of SM applied to human skin explants

Skin from a breast reduction on a 40-year-old white female (skin sample 8) was exposed to 0, 0.03, 0.1, 0.3, and 1.0% SM in methylene chloride (each 10 ul) in order to produce a dose-response curve. The explants were organ-cultured for 24 hr at 37 C, sectioned, and the number of paranuclear vacuoles counted as described above. Figure 1 shows our results. There was a straight-line relationship between the dose of SM and the number of vacuolated cells in the range of SM dosage.

The skin samples from different human beings show some variation in susceptibility to SM toxicity (Table 1). Such variation may be genetically and phenotypically determined. The human population is very heterogeneous. Also, variations in the thickness of the epidermal layer and its content of sebum exist. Paranuclear counts on diluent-exposed should always be made to establish a baseline.

Effect of storage at 4 C on the number of paranuclear vacuoles in unexposed human skin explants

Human skin explants from various sources were stored in the refrigerator (4 C) in covered Petri dishes for 1 to 15 days bathed in our standard medium RPMI 1640 containing penicillin and streptomycin. One skin specimen was stored at room temperature (23 C) for 22 hr.

Before storage, the subcutaneous fat was removed, and the skin was cut into pieces that fit into a standard 10-cm Petri dish. The medium (5 to 10 times the volume of the explant) was added so that the surfaces of the explants were not covered, but exposed to air.

On the days listed in Table 1, two explants, each 1.0 cm^2 in size, were gently rocked at 37 C for 24 hr in fresh medium in 5% CO_2--95% O_2. Then glycol methacrylate tissue sections were prepared, and the paranuclear vacuoles in two tissue sections across the center of each explant were counted.

Although variations among the skin specimens occurred, Table 1 clearly indicates that human skin survives well in the refrigerator for at least 3 days.

Effect of storage at 4 C on the number of paranuclear vacuoles in human skin explants exposed to SM

Table 1 lists six experiments on the effect of storage at 4 C on the sensitivity of human skin explants to SM. In Experiments 1 and 6, storage increased the sensitivity to SM, whereas in Experiments 4 and 5, storage had relatively little effect on the sensitivity to SM. In Experiments 2 and 3, the 0.2% SM produced a maximal number of vacuoles in specimens from all days of storage. The maximal number of vacuoles per centimeter of skin is 600 to 1000, depending on the skin specimen. Differences in this range are probably not significant.

These findings indicate that storage has more adverse effects on some skin specimens than on others. Nevertheless, stored human skin specimens can clearly be used to evaluate protective ointments and decontaminating agents, as long as the appropriate controls are included.

These studies suggest that cadaver skin would also be satisfactory, if it is obtained within a day or two of death.

Caveat

We are recounting the slides used to create Table 1 because there seem to be two types of vacuoles: one that surrounds the nucleus, which is found much more frequently in stored skin, and one that indents the nucleus on one side which is found much more frequently in SM-treated skin.

Effect of 4- and 24-hr incubation on the number of paranuclear vacuoles in unexposed human skin explants

Human skin explants (1.0 cm^2) were stored in the refrigerator at 4 C for 0 to 15 days. They were incubated in RPMI medium and 5% CO_2--95% O_2 for either 4 or 24 hr. The number of paranuclear vacuoles in each explant was then counted and this number was compared to the number found in non-incubated control explants.

Results are presented in Table 2. Incubation for 4 or 24 hr definitely decreased the number of paranuclear vacuoles in skin specimens stored in the refrigerator for 3 to 15 days.

The reason for this "recovery" remains to be investigated. Rough counts on the number of pyknotic nuclei in three of the experiments (Nos. 3, 4 and 5 of Table 2) (data not shown) suggest that vacuolated cells may become pyknotic cells without vacuoles during the incubation period. In other words, the number of pyknotic cells increased as the number of paranuclear vacuoles decreased. Cell lysis may be another reason for the disappearance of vacuolated epidermal cells during incubation. We did not ascertain whether or not such lysis occurs, because total epidermal cell counts are difficult to make. (The keratin layer of human skin, which contains numerous dead cells, is much thicker than that of fur-bearing laboratory mammals.)

Finally, some of the cells with paranuclear vacuoles may recover, and the vacuoles disappear. We believe this last possibility to be unlikely, because the appearance of these vacuoles under the electron microscope suggests irreversible damage to the nucleus (1).

Effect of 4-hr preincubation of human skin explants on the toxicity of SM applied subsequently

Human skin explants (stored 2 to 15 days) were preincubated (in duplicate) for 4 hr at 37 C, exposed to 0.2% SM (10 ul), and incubated for 24 additional hours (Table 3). With four sets of explants, the SM produced one-third to one-half the number of paranuclear vacuoles in the preincubated group than it produced in the group that was not preincubated. However, many sets were unchanged by preincubation, and one set showed substantially more vacuoles when similar comparisons were made.

These results suggest that preincubation of human skin explants has variable effects on the number of paranuclear vacuoles produced by a subsequent application of SM.

[^{14}C]Leucine incorporation test

The details of our first experiment have already been published (1). In brief, we incubated for 24 hr 1.0-cm^2 human skin explants with [^{14}C]leucine (0.25 uCi/ml of culture medium). Caucasian skin from an amputated leg was used. The proteins in the skin were precipitated, washed, solubilized, and digested. Then the radioactivity of the incorporated [^{14}C]leucine was read in a scintillation counter (see reference 1).

A straightline relationship was found between the decrease in [^{14}C]leucine incorporation and the dose of SM applied (10 ul to the surface of the skin explant) in the range of 0.1 to 1.0% SM (Figure 2). The effect of SM concentrations below 0.1% was not always detectable (Figure 2).

This experiment was repeated with Caucasian skin from a face lift on a 45-year-old female. Identical results were obtained. Dosages of 0.01 and 0.03% SM incorporated [^{14}C]leucine at almost control levels.

Comment. The [^{14}C]leucine incorporation is a valuable confirmatory test for the paranuclear vacuolization test. Both tests measure epidermal injury over the same concentration range of SM. Since the grinding and extraction of the skin is so time consuming, we recommend the paranuclear vacuolization test over the [^{14}C]leucine incorporation test.

Lysosomal enzymes, LDH, and protein released in vitro by full-thickness human skin explants

One-square centimeter full-thickness human skin explants were exposed in vitro to 1.0% SM and cultured for 24 hr. The culture fluids were collected, cleared by centrifugation, and frozen at -70 C until they were assayed for acid phosphatase, β-glucuronidase, β-galactosidase, lysozyme, LDH, and total protein.

No statistical differences were found between culture fluids from explants topically exposed to SM (or nitrogen mustard) and culture fluids from explants exposed to the diluent, methylene chloride (Table 4). The explants containing injured and dying epidermal cells, therefore, did not release greater amounts of these enzymes than explants containing uninjured (fully viable) epidermal cells. Since these enzymes are ubiquitous, those released by dying epidermal cells were evidently too small in amount to be detected.

Proteases released in vitro by full-thickness human skin explants

Culture fluids from 1.0-cm^2 full-thickness human skin explants, exposed in vitro to SM, were assayed for trypsin-like and chymotrypsin-like proteases with the synthetic peptide substrates, L-leucyl-glycyl-L-arginyl-amino-fluorocoumarin (LGA-AFC) and N-benzoyl-D,L-phenylalanine-β-naphthyl ester (BPN).

Table 5 presents our results. No statistical differences were found in the levels of trypsin-like proteases and chymotrypsin-like proteases in cul-

ture fluids from explants topically exposed to SM (or nitrogen mustard) and those in culture fluids from explants exposed to the diluent, methylene chloride.

LGA-AFC preparations were assayed with and without the plasmin inhibitor, aprotinin. It does not inhibit plasminogen activator, but does inhibit plasmin (18). These studies indicate that about 12% of the trypsin-like activity in the culture fluids was due to plasminogen-activator type of enzymes, and that 88% of the activity was due to plasmin and other aprotinin-inhibitable proteases.

Plasminogen activator

Culture fluids from full-thickness human skin explants treated topically with SM showed higher plasminogen activator (PA) activity than culture fluids of similar explants treated with the diluent $MeCl_2$ (Table 6). PA was measured with the ^{125}I-fibrin plate assay in the presence and absence of added plasminogen. After plasminogen was added, the increase in ^{125}I-peptide release is thought to be specific for PA.

These results suggest that SM causes the epidermal cells of the human skin explant to secrete (or release) PA. However, other interpretations are possible. (a) Epidermal cells may not be the major source of this PA. Fibroblasts may be the major source. (b) Culture fluids not containing skin explants sometimes showed greater release of ^{125}I-peptides when incubated 20 hr than those containing the explants (Table 6). In other words, inhibitors of fibrin digestion were present in human skin that entered the culture fluids during the 20-hr incubation time. (c) The tissue explants were not the only source of proteases. Evidently, the fibrin in the plate contained some, and the plasminogen also contained some (see the controls listed in Table 6). (d) The increase in ^{125}I-peptide release in the presence of added plasminogen identifies plasminogen activators, but many proteases can activate plasminogen. In other words, this assay method does not specifically identify the urokinase-type PA and tissue-type PA described in the literature (14). Blockage by specific antibodies would be necessary to accomplish this (14).

Comment. Dilute SM, applied topically to human skin explants, increases the proteases that activate plasminogen. Such proteases are known to cause blister formation (13,14). The use of organ culture proves that this increase is not due to proteases from the infiltrating leukocytes and the extravasated serum produced *in vivo* by SM, because the SM was applied *in vitro* to normal skin explants removed from the circulation. Therefore, PA-like proteases of skin activated by SM probably contribute to the blister formation caused by SM. Other epidermal and mesenchyme proteases may also be involved (see 20,21). Such blisters are a major effect of SM on exposed military personnel.

Angiotensin-converting enzyme

ACE (reviewed in 16, 22-24) is an ectoenzyme bound to the luminal surface of blood vessel endothelial cells. Upon damage, these cells release ACE into the blood stream. It is also present in activated macrophages. Since SM damages the microvasculature of the skin, as evidenced by the resulting edematous response (25, 26), we assayed the ACE in culture fluids of full-thickness human skin explants exposed to SM.

Explants. Human skin explants exposed in vitro to SM (or HN_2) generally had the same or less ACE activity than did control explants exposed to $MeCl_2$ (Table 7). These results are readily explained by a small study of ACE activity in rabbit SM lesion explants: Culture fluids from rabbit 1-day SM lesions showed 3 to 4 times the activity of culture fluids from explants of normal skin or 6-day SM lesions (Table 8). The ACE activity seemed proportional to the extravasated serum concentration in the culture fluids (Table 8).

Sera. Sera and plasma from each of two rabbits had identical ACE activities and protein concentrations. One unit of ACE activity was found in a 1:40 dilution of serum when 100 ul was assayed. Since the average protein concentration of the two undiluted sera was 71 mg/ml, these sera contained 5.6 units of ACE activity per mg of protein. Rabbit serum showed a change in fluorescence during the 45-min incubation time of 0.5 to 3.3 units in approximately a straightline relationship from a 1:80 to a 1:10 dilution. Carmel et al (17) reported that human serum showed a similar straightline relationship.

Comment. Normal human skin contains appreciable quantities of serum. In fact, 80% of the serum albumin in the body is normally extravascular, with skin and muscle containing most of it (27, see 26).

The explants, naturally, have no active blood circulation. It is possible that under these in vitro conditions the vascular endothelial cells in the $MeCl_2$-exposed controls die as rapidly as they die in the SM-exposed explants. SM and HN_2 probably injure any remaining viable endothelial cells in the full-thickness skin explant, so that less, not more, ACE activity was often found in the culture fluids from such in vitro produced lesions.

Addendum: A small confirmatory experiment was performed with hippuryl-L-histidyl-L-leucine (HHL) as the substrate (28). Full-thickness 1.0-cm^2 explants of human skin (from a face lift on a 45-year-old white woman) were incubated overnight with HHL in RPMI 1640 at pH 7.3, and for 3 hr on the next day at pH 8.2. Before incubation, three explants were topically exposed to 1% SM, and three were exposed to the diluent $MeCl_2$. SM-treated explants released into the culture fluids exactly the same amount of hippuric acid as did the $MeCl_2$ control explants. (Details on the methodology are in reference 28.)

Recapitulation. Vascular endothelial injury produced in vitro by SM does not increase the ACE activity of the human skin explant in a detectable fashion. Much of the ACE activity in these explants seems to be from the serum that these explants contain.

STUDIES IN PROGRESS

Blister formation in organ-cultured human skin explants

In a pilot experiment, 1% SM (10 ul) was applied topically to a 1.0-cm^2 full-thickness human skin explant. After 48 hr of incubation at 37 C, the epidermis had separated in the area where the SM had been applied. With light microscopy on thin (2 um) glycol methacrylate-embedded tissue sections, the epidermis had apparently separated from its basement membrane (see 1), but electron microscopy must be done to confirm this impression.

We studied blister formation in the series of human skin specimens presented in Tables 1, 2, and 3, which were used to evaluate paranuclear vacuole formation in explants that were stored at 4 C for 1 to 15 days, exposed topically to 0.2% SM, and incubated for 24 hr. Most of these specimens showed no blister formation, but in several, stored 7 to 15 days, about 0.5 mm of the 10 mm of epidermis contained microblisters. In case No. 5, skin from a leg that was amputated for peripheral vascular disease, 0.2% SM caused microblisters more frequently and after less time in storage.

Comment. To establish a reliable model for blister formation produced by SM in human skin explants, many more studies must be performed. We shall use 1% SM (rather than 0.2% SM) and 48-hr (rather than 24-hr) incubation times at 37 C.

Chemotactic factors for granulocytes and macrophages in culture fluids from full-thickness 1.0-cm^2 explants of human skin exposed in vitro to SM

Culture fluids from 1.0-cm^2 full-thickness human skin explants, exposed in vitro to 1% SM, were assayed for chemotaxins by the methods described in 29-32; they are also described in Chapter 4 of reference 5.

In our first experiments, full-thickness human skin explants, exposed topically to the SM, released smaller (not greater) amounts of chemotactic factors (for both granulocytes and macrophages) than did normal skin explants exposed only to the $MeCl_2$ diluent. This finding was unexpected, because granulocytes and macrophages infiltrate SM lesions produced in vivo (4,25,33). Evidently, epidermal cells exposed to SM produce chemotactic factors early, while the cells are still alive. These factors are then destroyed or inactivated.

Many more studies must be done to identify these early chemotactic factors. At various times after the application of SM, we shall assay the culture fluids (with the help of collaborating laboratories) for leukotriene B_4, the complement component, C5a, and interleukin 1, all of which are potent chemotactic factors.

Phlogistic factors in culture fluids of full-thickness 1.0-cm^2 explants from human skin exposed in vitro to SM

The culture fluids will also be assayed for some of the inflammatory mediators causing vasodilation and vascular leakage: histamine, the C3a (and C5a) component of complement, and other eicosanoids. As described in our contract, these studies, and those listed above under "Chemotactic Factors," frequently involve collaboration with other laboratories.

CONCLUSION

During the first year of this contract, we developed our paranuclear vacuolization test and [^{14}C]leucine incorporation test, so that they can be used to test the efficacy of protective ointments (and decontaminants) on human skin exposed to SM.

We also made considerable progress in evaluating the early inflammatory mediators produced by SM in human skin. To date, the most important mediator (increased by the application of SM) is plasminogen activator, a proteolytic enzyme associated with blister formation (13,14).

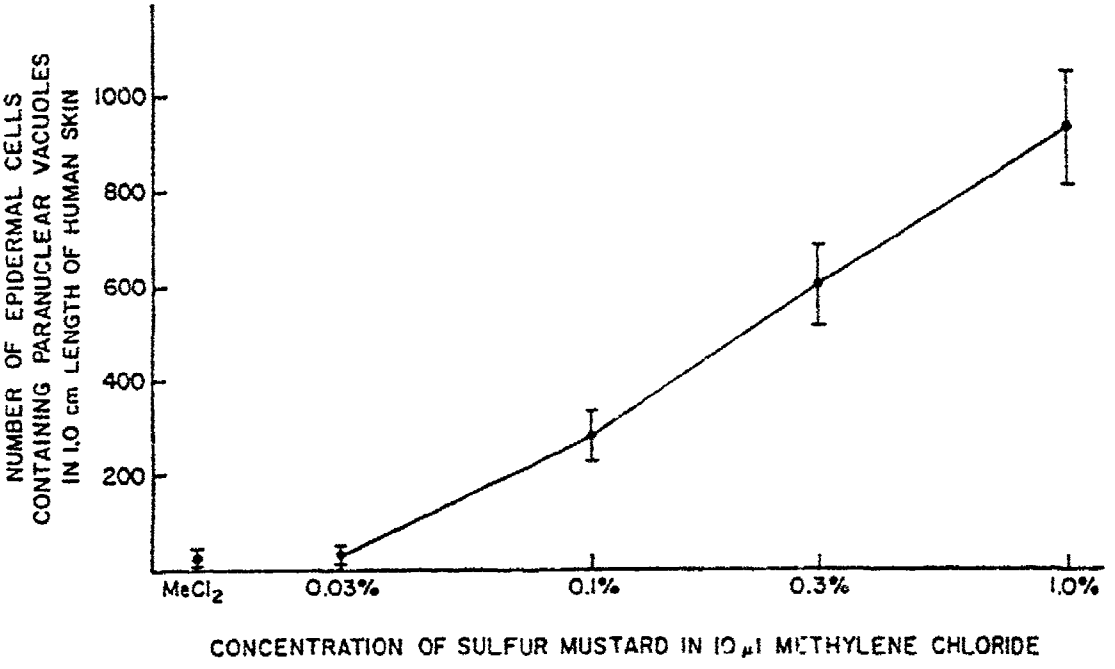

Figure 1. The effect of various concentrations of SM on the number of paranuclear vacuoles in epidermal cells of full-thickness human skin explants. One-cm^2 explants received a topical application of SM and were cultured for 24 hr. Then, centrally located tissue sections were prepared. The number of vacuolated epidermal cells was proportional to the concentration of SM in the range depicted. The means (and their standard errors or range) of two to four explants are presented.

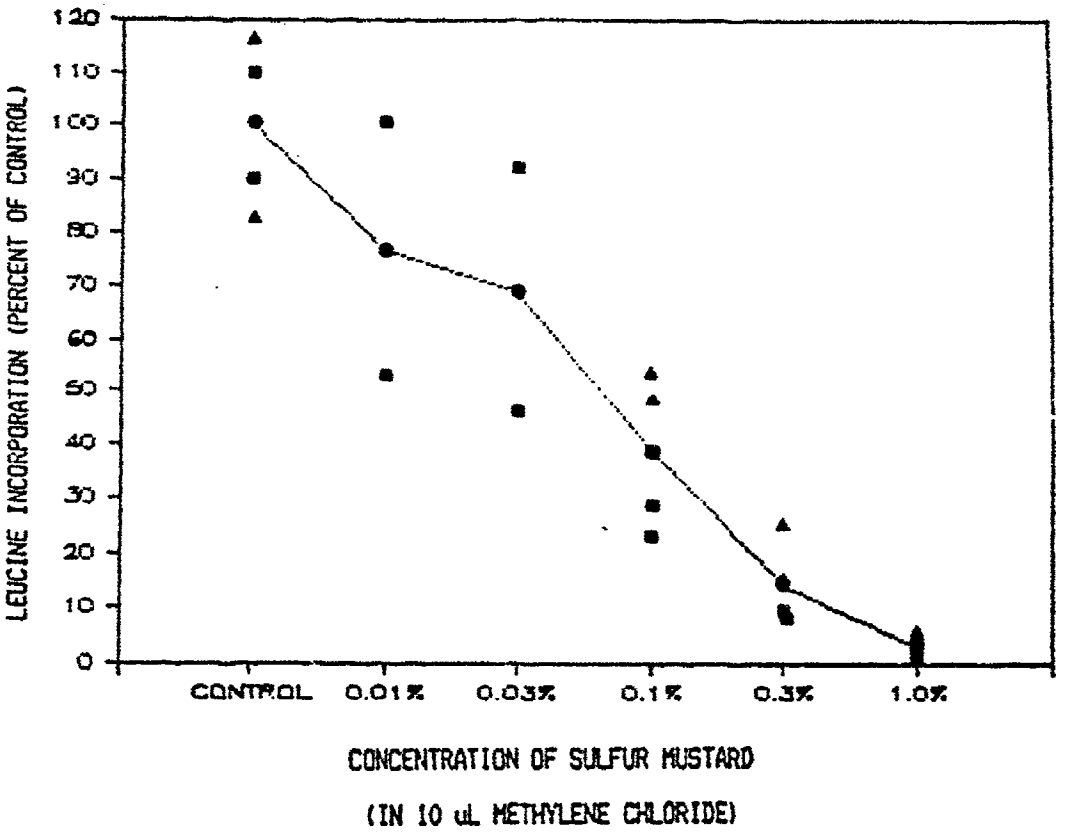

Figure 2. The effect of various concentrations of SM on protein synthesis in organ-cultured human skin. The amount of [^{14}C]leucine incorporation (during 24 hr in organ culture) by 1.0-cm^2 full-thickness explants of human skin was determined. Such incorporation is a measure of protein synthesis by the explant. A decrease in protein synthesis is a measure of injury (toxicity) to the explant. The lowest concentration of SM with distinct effects on every explant is 0.1%. Each explant is marked with a different symbol. $MeCl_2$ diluent (control), 0.1, 0.3, and 1.0% SM was applied to four explants (two from each of two patients); 0.01 and 0.03% concentrations were applied to two explants (from one of these patients). The line connects the means. ■ Explants from patient A; ▲ explants from patient B.

Table 1

Effect of storage of human skin explants at 4 C on the number of epidermal paranuclear vacuoles before and after the topical application of SM[a]

Skin Sample No. and Type of Skin	Days in Storage at 4 C	Number of Paranuclear Vacuoles in a Central 1.0-cm Tissue Section Across the Explant After 24-hr Incubation at 37 C	
		Controls[b]	0.2% SM[c]
1			
Normal breast	1 day		300 ± 6
skin, black	4 day	12 ± 1	740 ± 22
female, age 30	7 day	16 ± 4	900 ± 13
2			
Normal breast	0	11 ± 4	1090 ± 33
skin, white	5 day	70 ± 11	630 ± 32
female, age 27	9 day	36 ± 5	1040 ± 35
3	2 day	40 ± 5	860 ± 24
Normal breast	5 day	17 ± 2	880 ± 23
skin, black	8 day	15 ± 2	990 ±141
female, age 24	12 day	360 ± 14	1010 ± 30
	15 day	140 ± 20	1020 ± 66
4			
Normal leg	3 day	4 ± 1	410 ± 31
skin, white	6 day	7 ± 1	720 ± 15
female, age 79	10 day	8 ± 4	520 ± 74
5	1 day	10 ± 3	350 ± 5
Normal leg	4 day	14 ± 2	460 ± 18
skin, white	8 day	132 ± 4	410 ± 14
male, age 72	15 day	159 ± 5	390 ± 25
6			
Normal thigh	1 day	79 ± 16	280 ± 65
skin, white	2 day	11 ± 2	230 ± 49
female, age 27	3 day	14 ± 4	530 ± 27
	1 day at 23 C[d]	163 ± 6	330 ± 28

Footnotes on next page.

Footnotes to Table 1:

[a] The skin was received in our laboratory 2 to 4 hr after surgery. It was kept refrigerated at 4 C until used. SM was applied to the stored skin specimens within an hour of their removal from the refrigerator. Two explants were cultured for each value listed, and the paranuclear vacuoles were counted in two tissue sections from each explant. The four paranuclear counts were averaged and the standard errors or the means were listed.

[b] The controls received no topical application.

[c] In Experiment 6, 0.1% SM (rather than 0.2%) was applied to the surface of the explants.

[d] We plan to repeat this experiment with specimens from other sources.

Table 2

Effect of storage of normal human skin explants at 4 C
and subsequent 4-hr or 24-hr incubation at 37 C
on the number of paranuclear vacuoles in the epidermal cells

Skin Sample No. and Type of Skin	Days in Storage at 4 C	Number of Paranuclear Vacuoles in a 1.0-cm Tissue Section Across the Explant		
		0 hr	4-hr incubation	24-hr incubation
1	1	1 ± 1	3 ± 1	
Normal breast	4	62 ± 6	3 ± 1	12 ± 1
skin, black	7	71 ± 9	11 ± 1	16 ± 4
female, age 30				
2	0	3 ± 1	6 ± 1	11 ± 4
Normal breast	5	130 ± 20	57 ± 10	70 ± 11
skin, white	9	212 ± 60	68 ± 2	36 ± 5
female, age 27				
3	2	45 ± 18	4 ± 1	40 ± 5
Normal breast	5	115 ± 48	14 ± 5	17 ± 2
skin,	8	250 ± 29	51 ± 15	15 ± 2
black female,	12	220 ± 9	169 ± 14	360 ± 14
age 24	15	240 ± 10	102 ± 43	140 ± 20
4	3	6 ± 2		4 ± 1
Normal leg skin,	6	54 ± 4		7 ± 1
white female,	10	17 ± 18		8 ± 4
age 79				
5	1	3 ± 1	1 ± 1	10 ± 3
Normal leg skin,	4	59 ± 3	7 ± 1	14 ± 2
white male,	8	290 ± 7	170 ± 17	132 ± 4
age 72	15	250 ± 37	127 ± 21	159 ± 5
7	0	4 ± 1		39 ± 6
Normal breast	1	20 ± 7		29 ± 7
skin, white	2	89 ± 27		74 ± 30
female, age 36	3	148 ± 42		63 ± 7
8	0	4 ± 3		32 ± 4
Normal breast	5	220 ± 19		134 ± 4
skin, white				
female, age 40				

Legend on next page

Legend for Table 2:

Skin specimens in Experiments 1 to 5 were from the same patients as those listed in Table 1. Two explants, with two tissue sections from each, were evaluated for each value listed. The four paranuclear counts were averaged and the standard errors of the means were listed.

These data show that incubation for 4 or 24 hr decreased the number of paranuclear vacuoles in skin specimens stored at 4 C for 3 to 15 days.

Table 3

Effect of 4-hr preincubation of stored human skin explants on the toxicity of SM applied subsequently[a]

Skin Sample No. and Type of Skin	Days in Storage at 4 C	Number of Paranuclear Vacuoles in a 1.0-cm Tissue Section Across the Explant		
		No pre-incubation[b]	After 4-hr pre-incubation at 37 C[b]	Comments
1 Normal breast skin, black female, age 30	4	740 ± 22	440 ± 23	Decreased
	7	900 ± 13	480 ± 42	Decreased
2 Normal breast skin, white female, age 27	5	630 ± 32	630 ± 39	
	9	1040 ± 35	910 ± 25	
3 Normal breast skin, black female, age 24	2	860 ± 24	910 ± 20	
	5	880 ± 23	860 ± 119	
	8	990 ± 141	790 ± 141	
	12	1010 ± 30	950 ± 15	
	15	1020 ± 66	710 ± 15	Decreased
5 Normal leg skin, white male, age 72	4	460 ± 18	630 ± 179	
	8	410 ± 14	163 ± 9	Decreased
	15	390 ± 25	630 ± 22	Increased

[a] The skin specimens were from the same patients as those listed in Tables 1 and 2. SM (0.2%) was applied to both the preincubated and control explants. Then, they were incubated at 37 C for 24 hr, fixed, embedded in JB-4, sectioned and stained, and the number of paranuclear vacuoles determined. Two explants, with two tissue sections from each, were evaluated for each value listed. The four paranuclear counts were averaged and the standard errors of the means were listed.

[b] The explants that were not preincubated for 4 hr were kept at 4 C during that time.

Table 4

Lysosomal enzymes and LDH in culture fluids from 1.0-cm^2 human skin explants topically exposed *in vitro* to SM or nitrogen mustard (HN_2)

	Time in Culture	Acid Phosphatase	β-Glucuronidase	β-Galactosidase	Lysozyme ug/ml	Lactic Dehydrogenase mU/ml	Protein Concentration mg/ml
		nmol of p-nitrophenol produced per ml					
$MeCl_2$	3 hr	12.5 ±10.0	37.3 ±24.5	0	2.0 ±0.3	23.8 ±11.6	0.20 ±0.02
1% SM or 5% HN_2		17.4 ±12.6	34.7 ±22.9	0	2.3 ±0.6	22.6 ±11.8	0.19 ±0.02
$MeCl_2$	20 hr	16.4 ±11.1	85.0 ±49.5	0.65 ±0.65	2.8 ±0.3	37.2 ±18.4	0.44 ±0.05
1% SM or 5% HN_2		6.5 ±6.2	78.1 ±49.4	0	2.9 ±0.3	38.7 ±15.3	0.46 ±0.10

The explants were cultured either 3 or 20 hr after 10 ul of 1% SM or 5.0% nitrogen mustard (HN_2) was applied to their surfaces. The skin from three patients was used for the 3-hr cultures, and skin from six patients was used for the 20-hr cultures. The skin was usually provided by plastic surgeons from breast, abdominal or thigh reductions for obesity.

The means and their standard errors are listed. No statistically significant differences were found between explants exposed to SM (or HN_2) and those exposed to the $MeCl_2$ diluent.

Skin explants from one third of the donors were exposed to SM, and skin explan from two thirds of the donors to HN_2.

Table 5

Trypsin-like and chymotrypsin-like proteases in culture fluids from 1.0 cm^2 human skin explants topically exposed *in vitro* to SM or nitrogen mustard (HN_2)

Treatment	A Substrate for Trypsin-like Protease Activity (LGA-AFC) (Fluorescence units)	B Fluorescence Units in the Presence of Aprotinin (2.5 ug/ml)	Percent of Protease Activity Remaining (B/A x 100)	Chymotrypsin-like Protease Activity (BPN) (optical density units)
$MeCl_2$	47.5 ±10.1	6.8 ±2.0	14%	0.086 ±0.016
1% SM or 5% HN_2	41.1 ±10.5	4.5 ±0.6	11%	0.078 ±0.005

Substrates: LGA-AFC = leucyl-glycyl-arginyl-aminofluorocoumarin;
BPN = N-benzoyl-phenylalanine-B-naphthyl ester.

The full-thickness skin explants were cultured 20 hr after SM or HN_2 exposure before the culture fluids were collected. Culture fluids from three explants from each donor were pooled for each assay.

Five donors provided skin specimens for the trypsin-like protease assays, and six donors for the chymotrypsin-like protease assays. The skin specimens from one donor in each of these groups were exposed to HN_2 (instead of SM).

Aprotinin inhibits plasmin and trypsin, but does not inhibit plasminogen activator (References 9 and 19, and Chapter 6 of reference 5).

The means and their standard errors are listed. No statistical differences were found in the amounts of trypsin-like and chymotrypsin-like proteases released into the culture fluids by explants exposed to SM or HN_2 and the amount released by explants exposed to the $MeCl_2$ diluent.

Table 6

Plasminogen activator activity in culture fluids from 1.0-cm^2 human skin explants topically exposed *in vitro* to SM or nitrogen mustard (HN_2)

Skin Sample Number and Treatment	Amount of Plasminogen Added per well (ug)	Radioactivity of ^{125}I-peptides Released from ^{125}I-Fibrin by the Explant Culture Fluids in 1.5 hr at 37 C					
		Plasminogen present		Plasminogen absent		Difference	III Change due to added plasminogen in percent I/II x 100
		I cpm	percent E/C x 100[a]	II cpm	percent E/C x 100[a]	cpm	
9[b] $MeCl_2$	5	6398		6679		-281	96
1% SM		9108	142	6839	102	2269	133
10[b] $MeCl_2$	5	5398		8007		-2609	67
1% SM		6995	130	5780	72	1215	121
11[b] $MeCl_2$	5	7627		6981		646	109
5% HN_2		8585	113	6997	100	1588	123
11[c] $MeCl_2$	1.25	1697		1399		298	121
5% HN_2		3431	202	1386	99	2045	248
12[c] $MeCl_2$	1.25	3007		1725		1282	174
5% HN_2		22089	735	6812	395	15277	324
13[c] $MeCl_2$	1.25	2964		1664		1303	178
5% HN_2		15826	534	1697	102	14129	932
14[d] $MeCl_2$	2.5	442		423		19	104
1% SM		499	113	413	98	86	120
15[d] $MeCl_2$	2.5	463		362		101	127%
1% SM		682	147	421	116	261	162
Means and their standard errors:		265 ±84 (P = 0.004)[e]		136 ±37 (N.S.[e])		C = 122 ± 13 E = 270 ± 98 (P = 0.004[e])	

Footnotes on next page

Footnotes for Table 6

The explants were exposed to 1.0% SM (or $MeCl_2$ diluent) and incubated in the RPMI 1640 culture medium for 20 hr at 37 C. The culture fluids were frozen until assayed for plasminogen activator (PA). Then 50 ul was added to each well and incubated for 1.5 (or 3) hr.

The figures in columns I and III clearly indicate that SM increases the production (or release) of PA by the skin explants ($P = 0.004$). Plasminogen is the substrate for PA.

The variations in radioactivity released were due to fibrin plates having been made at different times and stored for different periods of time. The half-life of ^{125}I is 60 days.

[a] Experimental group (E) (SM or HN_2) divided by control group (C) ($MeCl_2$) times 100.

[b] Controls: There were 3318 ^{125}I-cpm (in 200 ul) when fresh RPMI medium with plasminogen (5 ug) was added to the fibrin plates and incubated 1.5 hr at 37 C. (No controls without plasminogen were included in this group of experiments.)

[c] ^{125}I-fibrin plates containing culture fluids from these explants were incubated 3 hr instead of 1.5 hr. Controls: There were 2084 ^{125}I-cpm (in 200 ul) when fresh RPMI medium, was added to the fibrin plates and incubated for 3 hr at 37 C. When plasminogen (1.25 ug) was included with this control, the counts were 4277/min.

[d] Controls: There were 1565 ^{125}I-cpm (in 200 ul) when fresh RPMI medium, without skin explants, was added to the fibrin plates and incubated for 1.5 hr at 37 C. When plasminogen (2.5 ug) was included with this control, the counts were 1971/min.

[e] Because of occasional very large values, the statistical tests based on normal distribution are not appropriate. Therefore, the nonparametric sign test, based on the binomial distribution, was used (34). Specifically, with eight control-experimental (C-E) pairs, the probability of E being greater than C eight times is 0.004 (and seven times is 0.03).N.S. = no significant difference.

Table 7

Angiotensin-converting enzyme activity in culture fluids from 1.0-cm^2 human skin explants topically exposed *in vitro* to SM or nitrogen mustard

Skin Sample Number and Treatment	Time After SM Exposure at Which CFs Were Collected (hr)	ABz-Gly Released		Comments	
		Fluorescence units[a]	Percent (E/C x 100)	Sample size (ul)	Incubation time with substrate for ACE
11 Nothing 1% SM	5	2.10 2.10	100	2000	4 hr
14 $MeCl_2$ 1% SM	4	0.06 0	0	50	45 min
16 $MeCl_2$ 5% HN_2	3	0.01 0.01	100	50	45 min
17 $MeCl_2$ 5% HN_2	3	0.25 0.20	80	100	2 hr
18 $MeCl_2$ 5% HN_2	3	0.05 0	0	100	2 hr
Mean and its standard error:			56 ±23% ($P = 0.07$)[b]		
9 $MeCl_2$ 1% SM	20	0.63 0.60	95	500	45 min
10 $MeCl_2$ 1% SM	20	0.51 0.49	94	500	45 min
14 $MeCl_2$ 1% SM	20	0.03	67	50	45 min
16 $MeCl_2$ 5% HN_2	20	0.05 0.03	60	50	45 min
17 $MeCl_2$ 5% HN_2	20	1.40 0.80	57	100	45 min
18 $MeCl_2$ 5% HN_2	20	0.25 0.15	60	100	45 min
Mean and its standard error:			72 ±7% ($P = 0.004$)[b]		

Footnotes for Table 7

[a] Fluorescent units: In our experiments, one fluorescent unit was equivalent to 1.5 nmol of ABz-Gly released from the ACE substrate, o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (17). ACE activity was the difference in the fluorescent units produced by a lesion culture fluid incubated with the substrate without EDTA and the units produced with the substrate in the presence of EDTA (which inactivates ACE) (see Materials and Methods).

[b] The matched-paired Student's t-test was used.

Table 8

Angiotensin-converting enzyme activity in culture fluids from 1.0-cm^2 rabbit SM lesion explants and control skin

Source of CFs	ABz-Gly Released in 45 min by 100 ul CFs (units)[b]	Protein Concentration (mg in 100 ul)	ABz-Gly Released per mg of Protein (units)[b]	Contribution of Serum to ACE Activity in Lesions[a] (units)[b]
Normal skin	A 0.28 ±0.05	0.05 ±0.005	D 5.8 ±0.8	0.24 (86%)
1-day SM lesion	B 1.01 ±0.07	0.16 ±0.014	E 6.2 ±0.4	0.76 (75%)
6-day SM lesion	C 0.32 ±0.08	0.10 ±0.007	F 3.4 ±0.8	0.48 (150%)

The means and their standard errors are listed. Culture fluids (CFs) of lesions and of normal skin from each of five rabbits were assayed. When the matched-paired Student's t-test was used: A vs. B, $P < 0.004$; A vs. C, N.S.; B vs. C, $P < 0.004$; D vs. E, N.S.; D vs. F, $P < 0.04$; E vs. F, $P < 0.004$.
N.S. = not statistically significant.

[a] We assumed that the protein in the culture fluids from the lesions (and normal skin) is 85% serum protein (26,35). One milligram of serum protein contained 5.6 units of ACE activity.

[b] In our experiments, one fluorescent unit was equivalent to 1.5 nmol of ABz-Gly released from the ACE substrate, o-aminobenzoylglycyl-p-nitro L-phenylalanyl-L-proline (17). ACE activity was the difference in the fluorescent units produced by a lesion culture fluid incubated with the substrate without EDTA and the units produced with the substrate in the presence of EDTA (which inactivates ACE) (see Materials and Methods).

LITERATURE CITED

1. Moore KC, Schofield BH, Higuchi K, Kajiki A, Au K-W, Pula PJ, Bassett DP, Dannenberg AM Jr: Two sensitive *in vitro* monitors of chemical toxicity to human and animal skin (in short-term organ culture): I. Paranuclear vacuolization in glycol methacrylate tissue sections. II. Interference with [^{14}C]leucine incorporation. J Toxicol-Cutaneous & Ocul Toxicol 1986, 5: 285-302.

2. Karnovsky MJ: A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 1965, 27: 137A.

3. Vogt RF Jr, Hynes NA, Dannenberg AM Jr, Castracane S, Weiss L: Improved techniques using Giemsa stained glycol methacrylate tissue sections to quantitate basophils and other leukocytes in inflammatory skin lesions. Stain Technol 1983, 58: 193-205.

4. Kajiki A, Higuchi K, Nakamura M, Liu LH, Pula PJ, Dannenberg AM Jr: Sources of extracellular lysosomal enzymes released in organ-culture by developing and healing inflammatory lesions. J Leukocyte Biol 1988, 43: 104-116.

5. Dannenberg AM Jr: Final Report, Contract No. DAMD17-80-C-0102. Pathogenesis and Treatment of Skin Lesions Caused by Sulfur Mustard: Inflammatory Mediators and Modulators Released from Organ-cultured Inflammatory Lesions Produced in Vivo in Rabbit Skin by Sulfur Mustard. (Currently being revised for final distribution.)

6. Huseby RM, Clavin SA, Smith RE, Hull RN, Smithwick EL Jr: Studies on tissue culture plasminogen activator. II. The detection and assay of urokinase and plasminogen activator from LLC-PK$_1$ cultures (porcine) by the synthetic substrate N-benzyloxycarbonyl-glycyl-glycyl-arginyl-4-methoxy-2-naphthylamide. Thromb Res 1977, 10: 679-687.

7. Nieuwenhuizen W, Wijngaards G, Groeneveld E: Fluorogenic peptide amide substrates for the estimation of plasminogen activators and plasmin. Anal Biochem 1977, 83: 143-148.

8. Smith RE, Bissell ER, Mitchell AR, Pearson KW: Direct photometric or fluorometric assay of proteinases using substrates containing 7-amino-4-trifluoromethylcoumarin. Thromb Res 1980, 17: 393-402.

9. Higuchi K, Kajiki A, Nakamura M, Harada S, Pula PJ, Scott AL, Dannenberg AM Jr: Proteases released in organ culture by acute dermal inflammatory lesions produced in vivo in rabbit skin by sulfur mustard: Hydrolysis of synthetic peptide substrates for trypsin-like and chymotrypsin-like enzymes. Inflammation 1988, 12: 311-334.

10. Dannenberg AM Jr, Bennett WE: Hydrolytic enzymes of rabbit mononuclear exudate cells. I. Quantitative assay and properties of certain proteases, nonspecific esterases and lipases of mononuclear and polymorphonuclear cells and erythrocytes. J Cell Biol 1964, 21: 1-13.

11. Rojas-Espinose O, Arce-Paredez P, Dannenberg AM Jr, Kamenetz RL: Macrophage esterase: Identification, purification and properties of chymotrypsin-like esterase from lung that hydrolyses and transfers nonpolar amino acid esters. Biochim Biophys Acta 1975, 403: 161-179.

12. Ossowski L, Quigley JP, Kellerman GM, Reich E: Fibrinolysis associated with oncogenic transformation: Requirement of plasminogen for correlated changes in cellular morphology, colony formation in agar, and cell migration. J Exp Med 1973, 138: 1056-1064.

13. Hashimoto K, Shafran KM, Webber PS, Lazarus GS, Singer KH: Anti-cell surface pemphigus autoantibody stimulates plasminogen activator activity of human epidermal cells. A mechanism for the loss of epidermal cohesion and blister formation. J Exp Med 1983, 157: 259-272.

14. Morioka S, Lazarus GS, Jensen PJ: Involvement of urokinase-type plasminogen activator in acantholysis induced by pemphigus IgG. J Invest Dermatol 1987, 89: 474-477.

15. Ploug J, Kjeldaard NO: Urokinase - An activator of plasminogen from human urine. Isolation and properties. Biochim Biophys Acta 1957, 24: 278-282.

16. Kelley J: Lavage angiotensin-converting enzyme as a marker of lung injury. Am Rev Respir Dis 1988, 137: 531-534.

17. Carmel A, Ehrlich-Rogozinsky S, Yaron A: A fluorimetric assay for angiotensin-I converting enzyme in human serum. Clin Chim Acta 1979, 93: 215-220.

18. Carmel A, Yaron A: An intramolecularly quenched fluorescent tripeptide as a fluorogenic substrate of angiotensin-I-converting enzyme and of bacterial dipeptidyl carboxypeptidase. Eur J Biochem 1978, 87: 265-273.

19. Dano K, Reich E: Plasminogen activator from cells transformed by oncogenic virus. Biochim Biophys Acta 1979, 566: 138-151.

20. Ikeda S, Manabe M, Muramatsu T, Takamori K, Ogawa H. Protease inhibitor therapy for recessive dystrophic epidermolysis bullosa: In vitro effect and clinical trial with camostat mesylate. J Am Acad Dermatol 1988, 18: 1246-1252.

21. Chapman HA Jr, Reilly JJ Jr, Kobzik L. Role of plasminogen activator in degradation of extracellular matrix protein by live human alveolar macrophages. Am Rev Respir Dis 1988, 137: 412-419.

22. Friedland J, Silverstein E: Properties of soluble and particulate angiotensin-converting enzymes of rabbit lung, induced macrophages and serum. Int J Biochem 1983, 15: 1337-1343.

23. Erdos EG, Skidgel RA: The angiotensin I-converting enzyme. Lab Invest 1987, 56: 345-348.

24. Okabe T, Yamagata K, Fujisawa M, Watanabe J, Takaku F, Lanzillo JJ, Fanburg BL: Increased angiotensin-converting enzyme in peripheral blood monocytes from patients with sarcoidosis. J Clin Invest 1985, 75: 911-914.

25. Dannenberg AM Jr, Pula PJ, Liu L, Harada S, Tanaka F, Vogt RF Jr, Kajiki A, Higuchi K: Inflammatory mediators and modulators released in organ-culture from rabbit skin lesions produced in vivo by sulfur mustard. I. Quantitative histopathology; PMN, basophil and mononuclear cell survival; and unbound (serum) protein content. Am J Pathol 1985, 121: 15-27.

26. Harada S, Dannenberg AM Jr, Kajiki A, Higuchi K, Tanaka F, Pula PJ: Inflammatory mediators and modulators released in organ culture from rabbit skin lesions produced in vivo by sulfur mustard. II. Evans blue dye experiments that determined the rates of entry and turnover of serum protein in developing and healing lesions. Am J Pathol 1985, 121: 28-38.

27. Katz J, Sellers AL, Bonorris G, Golden S: Studies on extravascular albumin of rats. In: Plasma Protein Metabolism: Regulation of Synthesis, Distribution and Degradation. Edited by MA Rothschild, T Waldmann. New York, Raven Press, 1970, pp. 129-154

28. Cushman DW, Cheung HS: Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem Pharmacol 1971, 20: 1637-1648.

29. Kreutzer DL, McCormick JR, Thrall RS, Hupp JR, Moore VL, Fink JN: Elevation of serum chemotactic factor inactivator activity during acute inflammatory reactions in patients with hypersensitivity pneumonitis. Am Rev Respir Dis 1982, 125: 612-614.

30. Kreutzer DL, Hupp JR, McCormick JR: Elevation of serum chemotactic factor inactivator activity during acute inflammatory reactions in rabbits and humans. Ann NY Acad Sci 1982, 389: 451-453.

31. Maderazo EG, Woronick CL: A modified micropore filter assay of human granulocyte leukotaxis. In: Leukocyte Chemotaxis. Edited by JI Gallin, PG Quie. New York, Raven Press, 1978, pp. 43-55.

32. Snyderman R: Chemotaxis of human and murine mononuclear phagocytes. In: Methods for Studying Mononuclear Phagocytes. Edited by DO Adams, PJ Edelson, H Koren. New York, Academic Press, 1981, pp. 535-547.

33. Vogt RF Jr, Dannenberg AM Jr, Schofield BR, Hynes NA, Papirmeister B: Pathogenesis of skin lesions caused by sulfur mustard. Fund Appl Toxicol 1984, 4: S71-S83.

34. Siegel S: Nonparametric Statistics for Behavioral Sciences: New York, McGraw-Hill Book Co., 1956.

35. Harada S, Dannenberg AM Jr, Vogt RF Jr, Myrick JE, Tanaka F, Redding LC, Merkhofer RM, Pula PJ, Scott AL. Inflammatory mediators and modulators released in organ culture from rabbit skin lesions produced in vivo by sulfur mustard. III. Electrophoretic protein fractions, trypsin-inhibitory capacity, α_1- and α_2-macroglobulin proteinase inhibitors of culture fluids and serum. Am J Pathol 1987, 126: 148-163.

ACKNOWLEDGMENTS

The following scientists, of our laboratory, collaborated in this research project: Dr. Masahiro Nakamura, Dr. Takafumi Yano, K. Gregory Moore and Peggy J. Pula.

We are grateful to Dr. Helen Abbey, of our Biostatistics Department, who advised us on the statistical treatment of our data; and to Ilse M. Harrop, for typing and editing the manuscript.

DISTRIBUTION LIST

1 copy	Commander US Army Medical Research and Development Command ATTN. SGRD-RMI-S Fort Detrick Frederick, MD 21701-5012
5 copies	Commander US Army Medical Research and Development Command ATTN. SGRD-PLE Fort Detrick Frederick MD 21701-5012
2 copies	Administrator Defense Technical Information Center ATTN. DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Commandant Academy of Health Sciences, US Army ATTN. AHS-CDM Fort Sam Houston, TX 78234-6100
1 copy	Dean, School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799